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VISUAL PIGMENTS OF RODS AND CONES IN A HUMAN RETINA

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SUMMARY

1. Microspectrophotometric measurements have been made of the photopigments of individual rods and cones from the retina of a man. The measuring beam was passed transversely through the isolated outer segments.

2. The mean absorbance spectrum for rods ($n = 11$) had a peak at $497{\cdot}6 \pm 3{\cdot}3$ nm and the mean transverse absorbance was $0{\cdot}035 \pm 0{\cdot}007$.

3. Three classes of cones were identified. The long-wave cones ('red' cones) had a λ_{max} of $562{\cdot}8 \pm 4{\cdot}7$ nm ($n = 19$) with a mean transverse absorbance of $0{\cdot}027 \pm 0{\cdot}005$. The middle-wave cones ('green' cones) had a λ_{max} of $533{\cdot}8 \pm 3{\cdot}7$ nm ($n = 11$) with a mean transverse absorbance of $0{\cdot}032 \pm 0{\cdot}007$. The short-wave cones ('blue' cones) had a λ_{max} of $420{\cdot}3 \pm 4{\cdot}7$ nm ($n = 3$) with a mean transverse absorbance of $0{\cdot}037 \pm 0{\cdot}011$.

4. If assumptions are made about the length of cones and about pre-receptoral absorption, it is possible to derive psychophysical sensitivities for the cones that closely resemble the appropriate π mechanisms of W. S. Stiles.

5. If assumptions are made about the length of rods and about pre-receptoral absorption, however, the psychophysical sensitivity derived for the rods is considerably broader than the C.I.E. scotopic sensitivity function.

INTRODUCTION

For several years now microspectrophotometers have reached advanced stages of design, and numerous applications of these instruments to the measurement of spectral absorbance curves of animal photoreceptors have been made in various laboratories. It is surprising, therefore, that nothing further appears to have been done with human retinas during the 15 years that have elapsed since the pioneering work of Marks, Dobelle & MacNichol (1964) and of Brown & Wald (1964).

Marks *et al.* (1964) showed records from one 'red' and one 'blue' human cone, which had λ_{max} at 577 and 460 nm respectively, while Brown & Wald (1964), and Wald & Brown (1965), reported results from three 'red', four 'green' and two 'blue' cones, and from two rods. From their records they obtained the absorbance changes

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on bleaching (difference spectra), which were maximal at 555–565, 525–530, 440–450 and 505 nm respectively.

These early recordings were made axially, with the attendant uncertainty whether the measuring beam had passed through the chosen outer segment without invading other cells. Contemporary, but unpublished, transverse observations that were free from this uncertainty were made by Liebman, but were not reported until 1972. Liebman found foveal cones with λ_{max} at 575–580 nm and at 535 nm and 'some evidence for a pigment of λ_{max} = 440 nm in end-on measurements of immediately parafoveal cones' (Liebman, 1972).

In his review of microspectrophotometry Liebman (1972) wrote, 'Human retinas can usually be obtained only under experimentally undesirable conditions of uncertain light exposure and pathology, or many hours after death. Human retinas inevitably must be examined only after severe light scattering changes have ensued. Unfortunately, almost none of the original data has even been shown in reports on primate pigments, and no mention has been made of the unacceptable experimental conditions that have been tolerated'. In view of these strictures and the special interest of human pigments, it is curious, both that the meagre data on human visual pigments has not been added to, and that no attempt has been made to obtain better material.

Through the co-operation of patient and surgeon at Moorfields Eye Hospital, we obtained a fresh dark-adapted human retina from which forty-eight different transverse records of foveal and parafoveal photoreceptors were obtained.

METHODS

The patient, a male Caucasian aged 46, had his right eye removed because of an intraocular tumour that was a malignant melanoma of the choroid. This had previously been treated by the application of a cobalt plaque in January, 1975. The evening before the operation on 27 July, 1977, the right eye was covered by a light-tight bandage, and this was not removed until commencement of the operation which was carried out under deep-red light in a blacked-out theatre. The illumination was provided by the standard operating lights over which ruby Cinemoid no. 14 filters were fitted. This filter has zero transmission between 430 and 590 nm, about 50% transmission at 630 nm and nearly 90% transmission at 700 nm. In addition, it has a low symmetrical transmittance band in the near ultra-violet rising to a peak of 13% at 375 nm.

Enucleation was completed at 10.35 a.m. and the eye was immediately placed in a light-tight Dewar flask containing ice and brought by road to Sussex University, where the first measurement of a photoreceptor was made at 1.25 p.m. The surgeon has informed us that the patient has normal colour vision in the remaining eye, while the patient himself has since confirmed that some time ago he had successfully passed all the Ishihara tests, and that he knew of no colour vision deficiencies in his family, either on his father's or his mother's side.

The eye was dissected under dim red light (Kodak safelight no. 2). An equatorial section of the globe was made, and the anterior half lifted away. The vitreous body was then removed from the posterior half without disturbing the retina, and the remaining eye cup placed in ice-cold mammalian Ringer solution of pH 7·1. The melanoma was visible as a dark patch about 3 mm in diameter. It did not encroach into the region of the fovea and optic disk.

Four pieces of retina (approximately 1 mm^2) were removed for investigation. The first, containing the fovea, was taken immediately after the dissection; the second, adjacent to the fovea, about 2·5 hr later; the third, 2–3 mm from the fovea, was taken next day, about 21·5 hr after the dissection; and the fourth, from a more peripheral region, about 2·5 hr after that. Throughout this time the eye cup was kept in darkness in the ice-cold Ringer solution. Each piece of retina was prepared on a slide in the way previously described for the rhesus monkey (Bowmaker,

Dartnall, Lythgoe & Mollon, 1978). All examinations of these preparations, and the lining up of individual cells for measurement, were done in infra-red with an image-converter for visualization.

The microspectrophotometer is of dual-beam design and is similar in most respects to that previously described by Liebman & Entine (1964) and by Liebman (1972). Some details of the instrument are given by Knowles & Dartnall (1977). At the start of each session the two equal beams of the microspectrophotometer were set at 2 × 1 μm, but they were adjusted equally as needed to suit the dimensions of the photoreceptors. All measurements were transverse and were generally made with the e-vector of the beams perpendicular to the outer segments in order to maximize their absorbance. The blue-sensitive cones (found only in the fourth preparation) and the rods kept their structure throughout, but the red- and green-sensitive cones progressively lost the original shape seen in the first preparation and later became spherical with little dichroism. However, previous experiments (Bowmaker, Loew & Liebman, 1975; Hárosi, 1975) have revealed no appreciable differences between 'parallel' and 'perpendicular' measurements as regards either λ_{max} or bandwidth.

The absorbance spectra were measured from 700 to 350 nm and then back to 700 nm, the double scan (taking 20 sec) showing that no significant bleaching occurred during the measurements (see Fig. 1). Deliberate bleaching of individual receptors was achieved by setting the grating monochromator to deliver white light. All four types of receptor were photolabile.

For the analysis of absorbance curves, four records out of a total of forty-eight were rejected outright for technical reasons. The remaining forty-four records were analysed individually by visually estimating, at 10 nm intervals, points lying midway within the noise band for both the absorbance and baseline traces. The baseline values were subtracted from the corresponding absorbance values, and the differences used to compute a normalized point curve. The λ_{max} was estimated by the method of Dartnall, Lander & Munz (1961) as amplified by Bridges (1967) and involved ten separate estimates based on the A_1 nomogram of Dartnall (Wyszecki & Stiles, 1967, p. 584). The precision of this analytical method for rhodopsin, and the reproducibility of absorbance measurements by microspectrophotometry, have been shown previously (Bowmaker, Loew & Liebman, 1975).

RESULTS

Typical spectra obtained from the four classes of photoreceptor found: a rod, a 'blue' cone (i.e. a blue-sensitive cone) and 'green' and 'red' cones are shown in Fig. 1.

In the first retinal sample, which included the fovea, three rods, six 'green' and ten 'red' cones were measured while the second, and adjacent, one yielded one rod, three 'green' and two 'red' cones. In the third sample, taken 2–3 mm from the fovea, four rods, one 'green' and six 'red' cones were measured, while in the fourth one there were three rods, three 'blue', two 'green' and four 'red' cones.

There were vastly more receptors in each preparation, many inaccessible to measurement, and because of this and the fact that we concentrated on cones, we cannot claim that the total of eleven rods and three 'blue', twelve 'green' and twenty-two 'red' cones are representative of the retinal population. Nor can we comment on any possible dependence on retinal location, though it is noteworthy that the only three 'blue' cones were found in the most peripheral preparation.

The eleven rod records had a mean transverse absorbance of $0{\cdot}035 \pm 0{\cdot}007$ and λ_{max} ranging from 491·5 to 505 nm with mean $497{\cdot}6 \pm 3{\cdot}3$ nm (S.D). Only one example each of the extreme λ_{max} values were obtained, the other nine records being closely grouped between 495 and 499·5 nm with a mean value of $497{\cdot}5 \pm 1{\cdot}5$ nm.

The three 'blue' cones had a mean transverse absorbance of $0{\cdot}037 \pm 0{\cdot}011$ and a

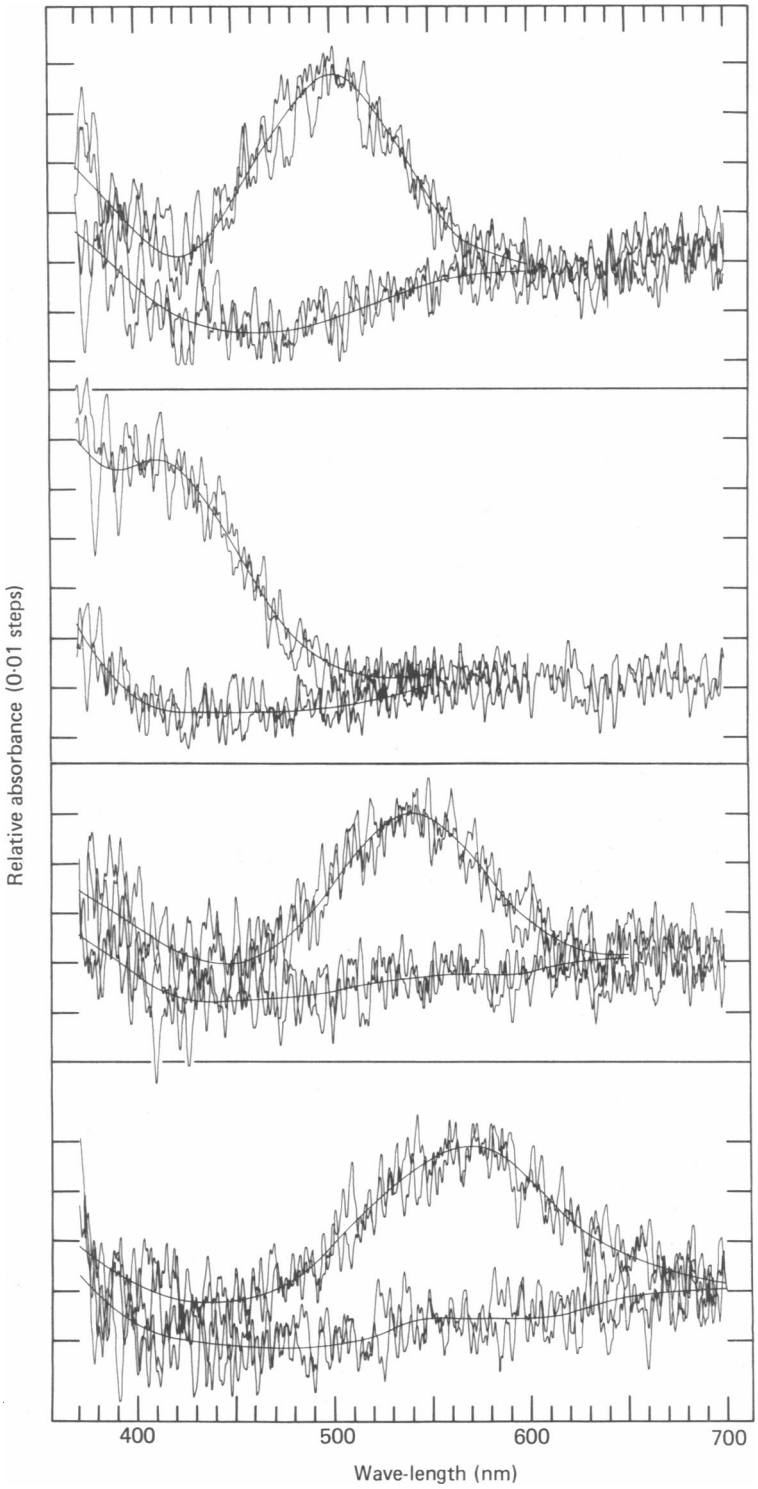

Fig. 1. Typical microspectrophotometric records from the outer segments of a rod (top frame) and of 'blue', 'green' and 'red' cones respectively. The continuous curves were drawn through the middle of the noise: the top curve in each example giving the relative absorbance of the visual pigment and the bottom curve being the instrumental baseline.

mean λ_{max} of 420·3 ± 4·7 nm. One record had λ_{max} = 415 nm (see Fig. 1) and the others were at 422 and 424 nm.

The twelve 'green' cones had a mean transverse absorbance of 0·032 ± 0·007. One record was rejected for λ_{max} determination because of base-line irregularities. The other eleven had a mean value of 533·8 ± 3·7 nm. Of these, two had λ_{max} between 528·5 and 529 nm and two between 539 and 539·5 (one of which is illustrated in Fig. 1). The other seven had mean λ_{max} of 533·6 ± 2·1 nm.

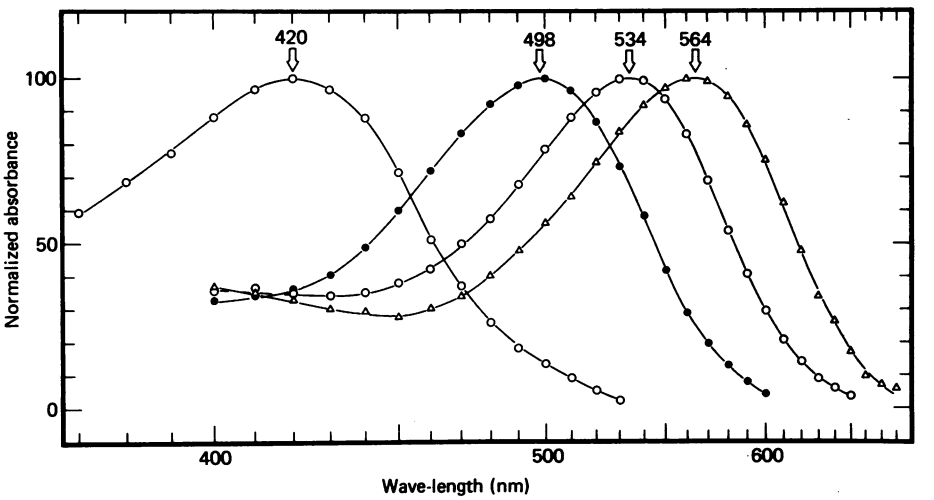

Fig. 2. The mean absorbance spectra of outer segments of the four classes of human photoreceptors. Curves labelled as follows: '498', mean of eleven rods; '420', mean of three blue-sensitive cones; '534', mean of eleven green-sensitive cones; '564', mean of nineteen red-sensitive cones.

Three of the twenty-two 'red' records obtained were rejected because of their low signal-to-noise ratio. The other nineteen had a mean transverse absorbance of 0·027 ± 0·005 and λ_{max} ranging from 554 to 569 nm with a mean at 562·8 ± 4·7 nm. Of these, four had λ_{max} values of 554–5 nm and three of 569 nm (one shown in Fig. 1). The remaining twelve had a mean of 563 ± 1·3 nm.

Fig. 2 gives the mean absorbance spectra for the four classes of photoreceptor. These curves have λ_{max} at 420 nm ('blue' cones), 498 nm (rods), 534 nm ('green' cones) and 564 nm ('red' cones), in close agreement with the values quoted above for the mean λ_{max} of individual records.

The abscissal axis in Fig. 2, though marked off in wave-lengths, is actually an equal frequency scale so that the longer wavelengths are 'cramped' relative to the shorter. According to the 'nomogram' hypothesis the shapes of all A_1-based pigments should be the same when plotted on this basis. It is clear, however, that the absorbance spectrum of the mean 'blue' cone is substantially broader than that of the others. The mean rod spectrum is an almost perfect nomogram curve, that of the mean 'green' cone also nearly perfect, and that of the mean 'red' cone only slightly narrower. The 'blue' cone curve, however, is undoubtedly broader and the results, taken as a whole, provide further evidence for the idea, first mentioned by Liebman

& Entine (1968), that the spectra of pigments, on a frequency scale, gradually become narrower as the λ_{max} advances to longer wave-lengths.

DISCUSSION

Comparisons with previous work

The present results are similar to those obtained in the recent microspectrophotometric investigation of the pigments of the rhesus monkey (Bowmaker *et al.* 1978). In this work the mean λ_{max} of the rod pigment was found to be $502 \pm 2{\cdot}7$ nm ($n = 25$). Comparison with the present human value of $498 \pm 3{\cdot}3$ nm ($n = 11$), suggests a real though slight difference between the rod spectra of the two species. On the other hand, the values for the 'green' cone, which for rhesus monkeys are $536 \pm 3{\cdot}5$ nm ($n = 42$), and for man $534 \pm 3{\cdot}7$ nm ($n = 12$); and for the 'red' cone, which for rhesus monkeys are $565 \pm 2{\cdot}5$ nm ($n = 40$) and for man $563 \pm 4{\cdot}7$ nm ($n = 19$) are probably the same within experimental error. These results are also in general agreement with the earlier results of Marks *et al.* 1964, Brown & Wald, 1964, and Wald & Brown, 1965, though it should be emphasized that until the present study only thirteen human receptors had been examined.

A significant difference between the data is apparent, however, with respect to 'blue' cones. None was recorded in our study of the rhesus monkey (Bowmaker *et al.* 1978), and previous data from three human 'blue' cones give λ_{max} of 460 nm (Marks *et al.* 1964), 450 nm (Brown & Wald, 1964) and 440 nm (Wald & Brown, 1965), whereas the present work places the λ_{max} between 415 and 425 nm. No immediate explanation for this discrepancy is apparent. The 'blue' cones reported by Marks *et al.* and by Wald & Brown had very low absorbances, however, even though the measurements were axial. Thus the values at λ_{max} were only 0·0017 in the single example of Marks *et al.* and only 0·009 and 0·025 in the two examples of Wald & Brown. These values are in marked contrast to the present mean *transverse* absorbance of 0·037.

Clustering

Of greater interest than the identity, or otherwise, of the mean λ_{max} values of the various visual pigments in man and rhesus monkey is the evidence, from both this investigation and the previous one (Bowmaker *et al.* 1978), that the λ_{max} values of the 'green' and 'red' cones are spread over an unexpectedly wide range. Only three 'blue' cones were found in the present work (and none in rhesus) so it is not known whether this applies to them also.

The λ_{max} values of the eleven 'green' cones measured in the present work ranged from 528 to 539 nm and those of the nineteen 'red' cones from 554 to 569 nm. In spite of the relatively low signal-noise ratios in primate records (Fig. 1, this paper; see also Bowmaker *et al.* 1978) we believe that these ranges (11 and 15 nm respectively) are significantly greater than one would expect from experimental error. The standard deviation of the ten estimates of λ_{max} made from each record (see Methods) did not exceed ± 5 nm, suggesting a standard deviation of about $\pm 1{\cdot}5$ nm for the mean value of an individual record. On this basis we should expect, if the pigment

were invariable, a range of some 5 nm, at most, in receptors of the same class, there being no evidence that the λ_{max} value of a receptor is affected by its precise orientation relative to the plane of polarization of the measuring beam (Bowmaker *et al.* 1975; Harosi, 1975).

It should be further noted that the λ_{max} values for the 'green' and 'red' cones are not distributed normally about the respective mean values, but occur discontinuously at certain spectral positions. Moreover, these positions correspond to those found in the rhesus monkey. Thus the rhesus work (Bowmaker *et al.* 1978) showed clustering at about 534 and 541 nm for 'green' cones, and at about 563 and 567 nm for 'red' cones. Similarly, the present human data suggest clustering at 528, 534 and 539 nm for 'green' cones, and at 555, 563 and 569 nm for 'red' cones.

The present work thus adds to the evidence suggesting that the λ_{max} values for a particular class of primate cone are neither invariable nor continuously distributed about the mean, but cluster in discrete spectral regions. This conclusion recalls the 'cluster hypothesis' advanced by Dartnall & Lythgoe (1965) from quite different evidence, namely the spectral distribution of extractable *scotopic* pigments in a wide variety of animals having vitamin A_1-based retinas, and by Bridges (1965) in fresh-water fishes with A_2-based retinas.

Comparison with psychophysical data

It is instructive to compare the mean absorbance spectra of the four types of photoreceptor with the human visual data. These are the C.I.E. scotopic sensitivity function which relates to the rods, and Stiles' 'π_3', 'π_4' and 'π_5' mechanisms which may be related to the 'blue', 'green' and 'red' cones.

The data have been obtained from Wyszecki & Stiles (1967). The C.I.E. scotopic sensitivities (log $V'(\lambda)$) are on an energy basis, and require the addition of log $1/\lambda$ to convert them to a quantum basis. The listed values of log π_3, log π_4 and log π_5, however, are already on the quantum basis. All these data have been 'normalized' to maxima of 2·0 ($= \log_{10} 100$) and are plotted as symbols in Fig. 3 against wave-number, an inset scale of wave-lengths being provided as well.

To put the comparisons on a quantitative basis two corrections must be applied to the photoreceptor data of Fig. 2. These are to allow for light lost prereceptorally, and to take account of the effective optical densities of the pigments.

Absorption of light by the pre-receptoral media takes place mainly in the lens and macula. The corrections for these selective light losses are listed in Wyszecki & Stiles (1967, pp. 216 and 218). The lens correction is appropriate for all four types of receptor, but the macular correction, while certainly applicable to the 'green' and 'red' cones, is not relevant for rods, for the scotopic function applies to observations at angles of not less than 5° from the fovea, which is outside the area of principal pigmentation. Its relevance to 'blue' cones is also problematical and is discussed below.

The need for the second correction follows from the supposition that visual response is a function of light absorbed by visual pigment (Dartnall & Goodeve, 1937) and hence depends on its effective optical density. The greatest possible effective density is, presumably, the axial density in the photoreceptor and, as before (Bowmaker *et al.* 1978), we have taken this to be 0·475 for the rods, and 0·525

for the 'red' and 'green' cones at their absorbance maxima, these values being based on the transverse measurements of density (Results). As regards the 'blue' cones, we have assumed them to have the same specific absorbance as the 'red' and 'green' cones ($0{\cdot}015\ \mu m^{-1}$) but have taken their length in the parafovea (where outer segments are shorter) to be $25\ \mu m$ on the basis of Polyak's (1941) measurements, and the axial density accordingly as 0·375.

The theoretical log sensitivity curves, as calculated from the photoreceptor data of Fig. 2 by applying the appropriate corrections for pre-receptoral absorption and for the axial densities of the pigments, are shown in Fig. 3 by the continuous-line curves.

The agreements of the curves calculated from the 'red' and 'green' cone data with the π_5 and π_4 mechanisms, respectively, are satisfactory bearing in mind the limitations of the data compared. Thus the divergence of the theoretical and actual sensitivities below about 490 nm is in the region where the variable effects of lens and macular pigmentation become important. Even so, the discrepancies are only in the order of 0·05–0·10 log units. Again, the divergencies at the long wave-lengths are where the pigment data are least reliable (a very small displacement of base line in the records due, say, to a slight cloudiness of the outer segment has a disproportionate effect in regions of low absorption).

The agreement between the sensitivity curve calculated from the 'blue' cone data and Stiles' π_3 (crosses in Fig. 3) is less good. This is not unexpected for not only do the remarks made above regarding variability in pre-receptoral absorption apply *a fortiori* at short waves but the mean 'blue' cone in Fig. 2 is based on only three records. If, instead of this curve, one of the same λ_{max} (420 nm) but of the 'nomogram'-shape appropriate to this spectral region (Knowles & Dartnall, 1977, p. 76) is used, the derived sensitivity curve (dotted in Fig. 3) is in better agreement with the π_3 data. It could be objected, however, that π_3 is not the appropriate function to compare with the extramacular blue cones and, indeed, π_3 does show a dip in sensitivity between 450 and 470 nm, due presumably to macular pigment (which has its maximum absorbance peak at about 460 nm). Fortunately, Stiles (1953) also measured π_1 (identical in terms of relative sensitivity with π_3 at short wavelengths) at 8° eccentricity, well clear of the macula lutea and this sensitivity function does not show a depression in the region of 460 nm (Fig. 3, squares). The discrepancy on the short-wave limb of the 'blue' sensitivities is probably due to variations in lens density between individuals. Wyszecki & Stiles (1967, p. 216) state 'sensory data show at short wave-lengths very large variations (of the order of 1 log unit at 400 nm), normally attributed to differing lens losses, and which occur even in the same age group'.

Finally we come to the comparison between the C.I.E. scotopic function (quantum basis) and the curve derived from the mean spectrum for the rods. This comparison is less equivocal than in the previous cases, for the rod spectra show little variation in the eleven records obtained and, in addition, are subject only to the uncertainty of the lens-absorption correction. Consequently, the clear indication in Fig. 3 that the C.I.E. function is markedly narrower than the curve derived from the rod spectrum assuming an axial absorbance of 0·475 is very significant. In fact, as shown by the dashed curve in Fig. 3, excellent agreement with the C.I.E. data is obtained by omitting the concentration-broadening effect.

In previous comparisons (Dartnall & Goodeve, 1937; Crescitelli & Dartnall, 1953) it was supposed that the narrowness of the C.I.E. function indicated a very low (i.e. less than 0·1) end-on absorbance for the rods.

The present measurements of the specific absorbance of rhodopsin make this assumption untenable. However, the same narrow function could result if it is assumed that only the portion of the pigment-bearing rod membrane close to the inner segment is visually effective.

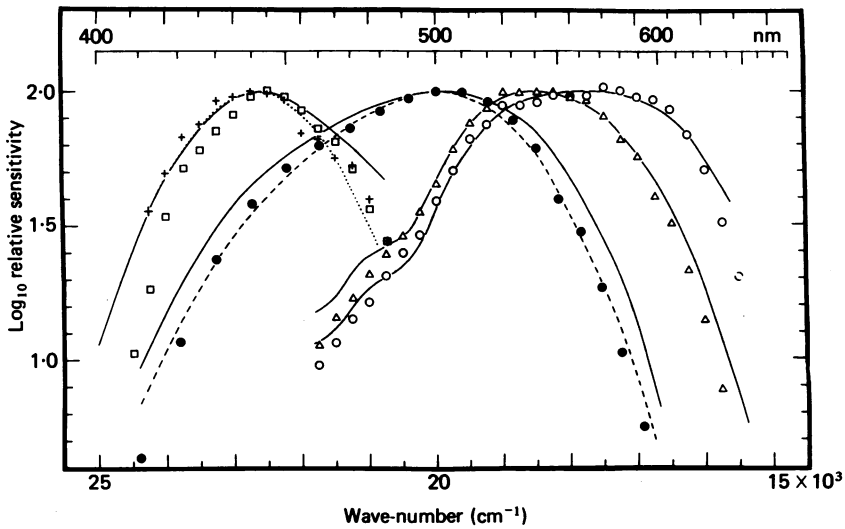

Fig. 3. Comparisons of the expected sensitivity functions as calculated for the four classes of receptor (continuous curves) with the π_5 (circles), π_4 (triangles), π_3 (crosses) and 8° eccentricity π_1 (squares) mechanisms of Stiles and the C.I.E. scotopic sensitivity (filled circles). The curves were calculated from the pigment data of Fig. 2 by allowing for lens absorption in all cases, and for macular absorption as well for the 'red' and 'green' cones and for axial absorbances as follow: 'red' and 'green' cones, 0·525; 'blue' cone, 0·375; rod, 0·475. The dotted curve was derived from a 'nomogram' curve of λ_{max} = 420 nm (see text). The dashed curve was calculated from the rod data after correction for lens absorption but assuming an axial absorbance of zero for the rod pigment.

This hypothesis can be supported by the structural differences between rods and cones. The membrane invaginations at the apical end of rod outer segments (Nilsson, 1964) migrate distally and become pinched off into disks isolated from the cell wall (Young, 1971). In contrast, in cones the invaginations remain open to the extracellular fluid along the entire length of the outer segment. If, on becoming pinched off, the disks lose the visual effectiveness of their contained pigment, then the effective density of pigment in rods would be very low, in contrast to that in cones where it would be equal to the density of the pigment measured axially through the outer segment.

In a personal communication, Dr W. S. Stiles has pointed out that the hypothesis is consistent with the fact that the rods show a much smaller Stiles–Crawford effect than cones. The effect in cones is thought to arise because light, in passing through the outer segment, is lost through the sides more for obliquely than for normally

incident light. If, in rods, only absorption close to the inner segment is effective, the difference between obliquely and normally incident light will be reduced and the expected Stiles–Crawford effect would be less.

However, although the hypothesis is attractive there are serious objections to it. First, it would oppose the idea that in order to increase absolute sensitivity the rod outer segment is lengthened to increase the pigment density: in some deep-sea fish living in a very dim photic environment the rod outer segments can be up to 200 μm long (Munk, 1966). Second, it would be counter to the results for other species where both the scotopic sensitivity and the rod visual pigment content is known. Thus the psychophysical scotopic sensitivity is closely matched by the *absorptance* spectrum of the rhodopsin in frog, where the end-on density is greater than 0·75 (Gordon & Hood, 1976) and in pigeon where it is about 0·5 (Bowmaker, 1977). In both these cases the lens is optically clear down to about 350 nm and so no pre-receptoral corrections have to be made.

A third objection is that on light stimulation a photocurrent can be measured along the total length of the outer segment of rat rods (Penn & Hagins, 1969), strongly suggesting that the entire outer segment is visually effective. This would appear to be confirmed by recent studies on single rod outer segments where it has been shown (Baylor, Lamb & Yau, 1979) that each element of the plasma membrane contributes approximately equally to the photocurrent.

The discrepancy between the C.I.E. scotopic sensitivity function and our derived sensitivity for rods is thus difficult to account for, especially so in view of the clear correlation of our derived psychophysical sensitivities of the three classes of cones with the π mechanisms of W. S. Stiles. There seems little doubt of the discrepancy, however, and an explanation is needed for it.

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